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## GENETIC VARIATION IN STRAINS OF DISCUS (*SYMPHYODON AEQUIFACIATA*) USING RAPD PCR

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Key words: band-sharing, DNA, morphology, PCR

### Abstract

The DNA of five strains of discus (*Symphyodon aequifaciata*) were compared by random amplified polymorphic DNA (RAPD PCR) using seven primers. Only two primers (ZG4 and ZG8) were suitable. Using primer ZG8, the red turquoise strain had five bands common to at least one fish of another strain and two bands common to all the fish in the red turquoise strain. Panda turquoise had one band in common with at least one fish of another strain and seven bands common to all panda turquoise. For royal red, these figures were five and one and for common brown they were three and one. Within the strains, the level of band-sharing was 0.83 in the red turquoise strain, 0.13 in the panda turquoise, 0.83 in the royal red and 0.75 in the common brown. Specific bands were found only with primer ZG8 in the common brown and panda turquoise strains. Using primer ZG4, very low variation was found between the royal red, cobalt blue and red turquoise strains. With both primers, the highest genetic variation was between the common brown strain and the others (0.33-0.83).

### Introduction

The Cichlidae is an important family in aquaculture (Trewavas, 1982). However, it is very difficult to identify the different cichlid species due to overlapping morphological characteristics as well as crossbreeding between species (Fryer and Iles, 1972).

Considerable advancements have been

made in the past thirty years in the fields of evolutionary population genetics and systematics in teleosts, which is probably the most complex vertebrate group both in number of species and in range of origins (Smithies, 1955; Raymonds and Weintraub, 1959; Greenwood et al., 1966).

Electrophoretic variations have been studied in many cichlid species, especially South African cichlids (Kocher et al., 1995; Sanja et al., 2003). McAndrew and Majumdar (1983) used electrophoretic markers on starch gel for various body tissues of the genera *Oreochromis* and *Tilapia*. Avtalion et al. (1975) used electrophoresis on polyacrilamide gel on blood serum to search for genetic markers in *Tilapia*. Degani and Levkovitch (1991) studied the specific enzyme variation of cichlid species cultivated in Israeli aquaculture by starch gel electrophoresis. Cichlidae are characterized by a wide variation in color and many lines have been distinguished by researchers. One of the most popular South American cichlids is the angelfish (*Pterophyllum scalare*), which has many varieties of color and shape (Degani et al. 1997; Goldberg et al., 2000). Degani et al. (2000) applied RAPD PCR in the study of genetic variations in Cichlidae in Israel.

The *Symphyodon aequifaciata* species (discus) of the Cichoasomine subfamily and the Cichlidae family (Lebiel, 1996) is found in the Amazon River in South America. This species differs from most other members of the Cichlidae family in the difficulty of breeding them and the amount of research and development invested in increasing the number of strains (Middleton, 1996a,b, 1997).

The purpose of the present research was to study genetic variations in five strains of discus (*Symphyodon aequifaciata*) found in aquaculture in northern Israel using the random amplified polymorphic DNA polymerase chain reaction (RAPD PCR) method. The importance of using this method lies not only in increasing the theoretical understanding of the genetic differences among discus strains but also in finding methods of identifying strains.

#### Materials and Methods

**Fish.** The fish used in this study were four strains (royal red, cobalt blue, panda turquoise and red turquoise) of the subspecies *Symphyodon aequifaciata aequifaciata* and one strain of the common brown subspecies *S. a. axelrodi*. These strains were imported to Israel and kept in the Migal laboratory.

**Sampling and DNA extraction.** The course of the experiment is shown in Fig. 1. Blood samples were taken from the heart of 20 randomly chosen fish of each strain, as described by Degani (1990), and stored in tubes with K3 EDTA at 70°C until DNA extraction. DNA was isolated from the blood, as described by Hillel et al. (1989), with a slight modification: 500 µl of 10 x SSC (0.15M Trisodium citrate, 1.5M NaCl) was added to 100 µl blood, then SDS (sodium dodecyl solution) was added to bring the final concentration to 1%. The mixture was extracted twice with equal volumes of phenol and chloroform. DNA was precipitated three times in 100% ethyl alcohol, rinsed in 70% ethyl alcohol and dissolved in TE (10mM Tris, pH = 8, 1 mM EDTA; Maniatis et al., 1982; Hillel et al., 1989).

**RAPD PCR.** DNA was amplified by a procedure modified from Hillel et al. (1989) by Degani et al. (2000) using seven 10-mer random unspecific primers (Operon, Alameda, CA, USA; Table 1). Each PCR reaction contained 50 ng genomic DNA, 1 µl 25 µM DNTPs mixture, 40 ng primer, 0.75 units of Taq DNA polymerase (Promega), 5 µl Taq buffer and 5 µl mM MgCl<sub>2</sub>. The reactions were carried out in a thermal cycler (Robocycler Gradient 96, Stratagene). The following program was used: heat-inactivation (94°C, 4 min), 40 cycles of denaturing (94°C, 1 min), annealing (35°C, 1 min), and extension (72°C, 1 min). The PCR product was electrophoresed on 2% agarose gel containing 3% ethidium bromide (Fig. 2).

**Analysis.** Only bands above 3 kb were analyzed. To assess genetic variations between fish, band-sharing (BS) was calculated as  $BS = 2(Nab)/(Na + Nb)$ , where BS = the BS between individuals a and b, Nab = the number of bands shared by a and b, Na = the total number of bands in a, and Nb = the total number of bands in b (Jeffreys and Morton, 1987; Wetton et al., 1987). When BS = 1, there was no variation (the bands were the same in both groups); when BS = 0, all the bands on the gels were different. The *d* test for differences between proportions (Parker, 1976) was used to calculate the significance of differences between BS.

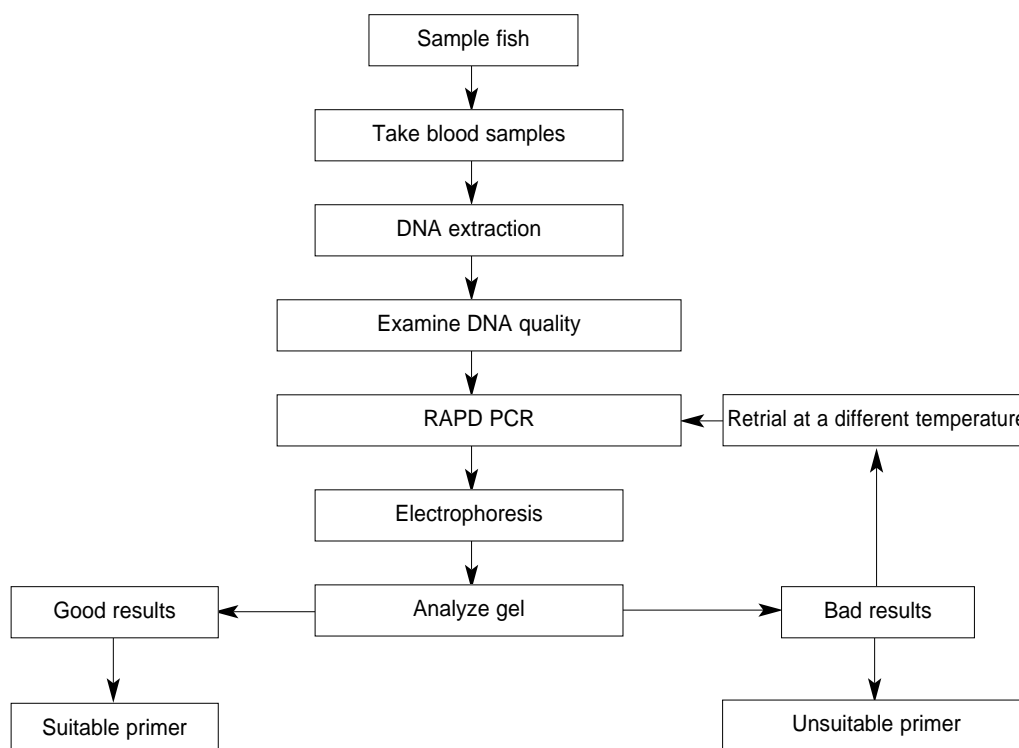


Fig. 1. Protocol for selecting suitable primers.

Table 1. Primers used in this study.

<i>Primer</i>	<i>Sequence</i>
K1	GGTAACGCC
K2	AAGGTCAGTGA
ZG2	GCACTGTCT
ZG4	GGAGCTGGC
ZG8	TGCCGAGCTG
G10	AGGGCCGTCT
I18	AGGTGACCGT

## Results

Only two (ZG4 and ZG8) of the seven primers were suitable for studying the DNA variations in question. The number of bands in the populations elucidated by the two suitable primers differed with each primer. Using ZG8, the number of bands common to one fish in one strain and at least one fish of another strain and the number of bands common to all the fish in a single strain were 2 and 5 in red turquoise, 1 and 7 in panda turquoise; 5 and 1 in royal red; and 3 and 1 in common brown, respectively (Table 2). Within the strains, the level of band-sharing was 0.83 in red turquoise, 0.13 in panda turquoise, 0.83 in royal red and 0.75 in common brown. Specific bands were found only with primer ZG8 in common brown and panda turquoise. The

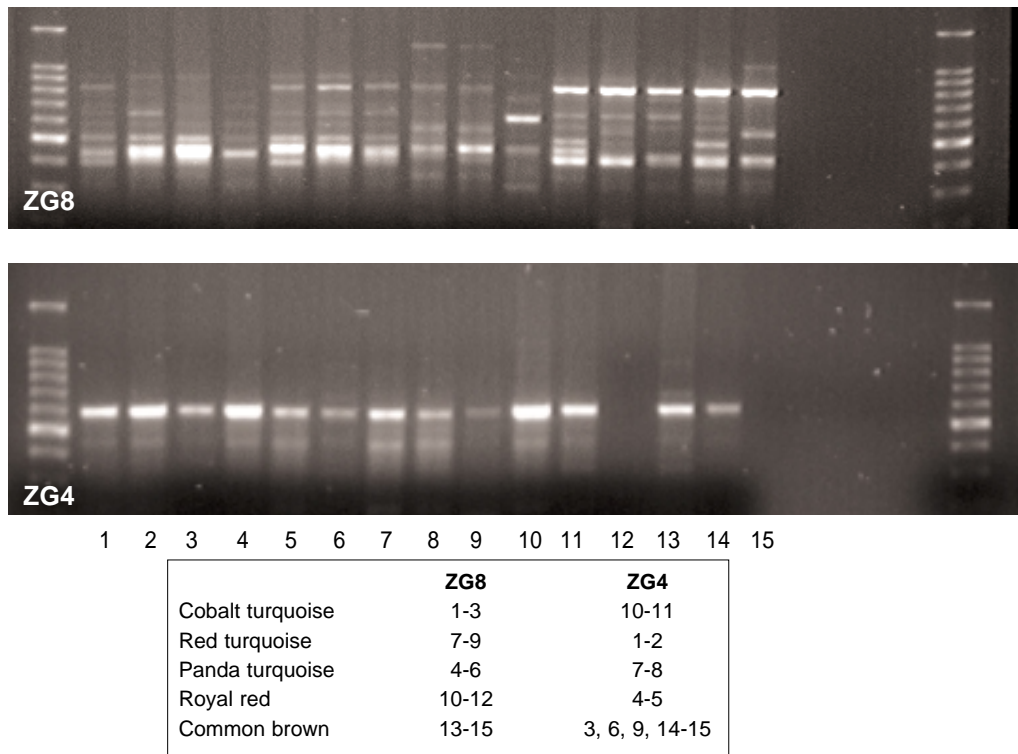


Fig. 2. Example of gel results using RAPD PCR with primers (a) ZG8 and (b) ZG4.

common bands found with primers ZG8 and ZG4 are shown in Table 3.

In both primers, the highest genetic variation was found between common brown and all the other strains (0.33-0.83; Table 4). Using ZG4, very low variation in band-sharing was found between royal red, cobalt blue and red turquoise, and between cobalt blue and red turquoise.

### Discussion

The genetic diversity of fish has been studied using various methods; the results and DNA polymorphism were affected by the method used to study the genetic diversity (see review by Yue et al., 2002a). The three methods were random amplified polymorphic DNA (RAPD), amplified fragment length polymor-

phism (AFLP), and microsatellites. These methods were used to estimate the genetic diversity in Asian arowana (*Scleropages formosus*). The multiplex ratio and marker index were higher for AFLP than for RAPD or microsatellites (Yue et al., 2002a). Many papers have been published on DNA marker systems for evaluating the genetic diversity of fishes (Bartfai and Orban, 2003; Yue and Orban, 2002a; Yue et al., 2002a,b).

The genetics of Cichlidae from Africa and Asia have been intensively studied (see review by Bank et al., 1989; Kocher et al., 1995; Lee et al., 1995; Yue and Orban, 2002b), but less attention has been paid to South American cichlids (Degani et al., 1997). The molecular methods used to intensively study genetic variations are mainly for African Cichlidae (see review by

Table 2. Band-sharing (BS) in five strains of discus (*Symphyodon aequifaciata*) as seen with primers ZG8 and ZG4.

Strain	No. bands found in this strain and in at least one fish of another strain	No. bands common to all fish in the strain	Band-sharing* of the two fish within a strain having the greatest variation
<i>Primer ZG8</i>			
Cobalt blue	4	3	0.57 <sup>a</sup>
Red turquoise	5	2	0.83 <sup>b</sup>
Panda turquoise	1	7	0.13 <sup>c</sup>
Royal red	5	1	0.83 <sup>b</sup>
Common brown	3	1	0.75 <sup>ab</sup>
<i>Primer ZG4</i>			
Cobalt blue	3	1	0.75 <sup>ab</sup>
Red turquoise	1	2	0.33 <sup>e</sup>
Panda turquoise	2	1	0.66 <sup>a</sup>
Royal red	2	1	0.66 <sup>a</sup>
Common brown	3	1	0.75 <sup>ab</sup>

Values with different superscripts differ significantly (*d* test; *p* < 0.05).

\*  $BS = 2(Nab)/(Na + Nb)$ , where BS = the variation within the strain; Nab = the number of bands shared by all the fish in the strain; Na = the total number of bands for the fish with the highest number of common bands, and Nb = the total number of bands for the fish with the lowest number of common bands.

Sanja et al., 2003). Moreover, most of the studies examined the differences between species rather than intraspecific variations. Since it is difficult to compare DNA fingerprinting (DFP) lanes on different gels, we compared bands only on single gels in which the DNA from the different strains had been mixed.

Color variations are well known among the cichlids and are used as systematic and genetic parameters in determining variations in this family (Fryer and Iles, 1972; Herzberg, 1978; Bank et al., 1989). Degani et al. (1997) studied strains of angelfish (*P. scalare*) that differ in color. Their findings show differences in pattern among the color strains using DFP

and RAPD PCR. Carleton and Kocher (2001), using Real-time PCR, demonstrated that cichlid species express different subsets of the available cone opsin genes through which the tuning of visual pigments is typically accomplished through changes in the opsin amino acid sequence. Within a given opsin class, changes at a few key sites control wavelength specificity. They found that variations in cichlid spectral sensitivity arose through the evolution of gene regulation rather than through changes in the opsin amino acid sequence. However, as far as we know, no previous study used DFP, which has the advantage of differentiating between

Table 3. Number of common bands in four strains of subspecies *Symphyodon aequifaciata aequifaciata* found with primers ZG8 and ZG4.

	<i>Red turquoise</i>	<i>Panda turquoise</i>	<i>Royal red</i>	<i>Common brown</i>
<i>Primer ZG8</i>				
Cobalt blue	6	4	4	2
Red turquoise		5	5	4
Panda turquoise			5	3
Royal red				3
<i>Primer ZG4</i>				
Cobalt blue	2	2	2	1
Red turquoise		2	3	1
Panda turquoise			3	1
Royal red				1

Table 4. Band-sharing\* in five strains of *Symphyodon aequifaciata* using primers ZG8 and ZG4.

	<i>Red turquoise</i>	<i>Panda turquoise</i>	<i>Royal red</i>	<i>Common brown</i>
<i>Primer ZG8</i>				
Cobalt blue	0.86 <sup>a</sup>	0.75 <sup>ab</sup>	0.71 <sup>ab</sup>	0.50 <sup>c</sup>
Red turquoise		0.63 <sup>bc</sup>	0.86 <sup>a</sup>	0.66 <sup>bc</sup>
Panda turquoise			0.71 <sup>ab</sup>	0.83 <sup>a</sup>
Royal red				0.42 <sup>c</sup>
<i>Primer ZG4</i>				
Cobalt blue	1.00 <sup>d</sup>	0.66 <sup>e</sup>	1.00 <sup>d</sup>	0.50 <sup>e</sup>
Red turquoise		0.66 <sup>e</sup>	1.00 <sup>d</sup>	0.50 <sup>e</sup>
Panda turquoise			0.66 <sup>e</sup>	0.33 <sup>f</sup>
Royal red				0.50 <sup>e</sup>

Values with different superscripts differ significantly (*d* test; *p* < 0.05).

\*  $BS = 2(Nab)/(Na + Nb)$ , where BS = the band-sharing between strains a and b, Nab = the number of bands shared by strains a and b, Na = the total number of bands in strain a, and Nb = the total number of bands in strain b.

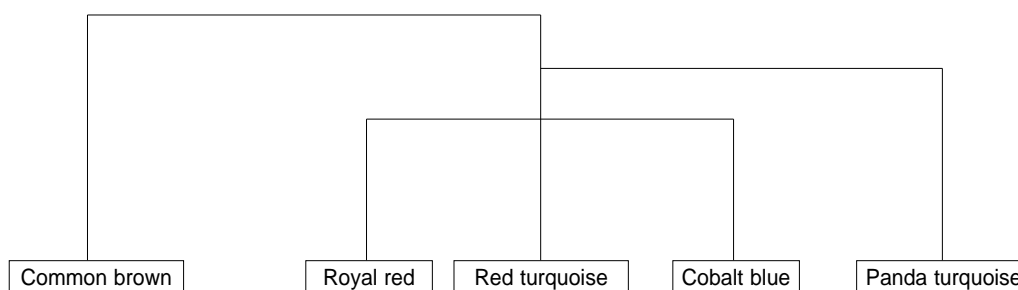


Fig. 3. Genetic relationship among four strains of discus of the subspecies *Symphyodon aequifaciata* and one strain of the subspecies *S. a. axelrodi* (common brown).

subspecies as well as between systematic groups (see review by Kocher and Stepien, 1997).

In our study, we found DNA variations using RAPD PCR. Hence, the method is useful not only for defining separate strains but also for determining the relationships between them. The differences may be due to morphological differences. Breeders obtain strains by selecting according to color, but such variations are the result of new mutations or an unknown existing gene. Fish vary in color in their natural habitats (Greenwood et al., 1966), perhaps due to intraspecific variation on a genetic level which DFP and PAPD PCR, as used in this study, can uncover. In this study, we examined strains of *S. aequifaciata* that had been developed by breeders. This species has many color patterns that were selected to develop new strains for the ornamental fish market. The genetic distance was found to be higher between strains of different subspecies than among strains developed from the same genetic origin (Fig. 3).

Other advantages of this method are that it can be used to determine the genotype of a strain in order to maintain its purity and to discover differences between new hybrid strains and their parent strains.

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